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(54) Cultivation of transformed microorganisms.

(57) A process for cultivating a microorganism transformed with a recombinant plasmid at least containing (a) a DNA fragment containing a promoter and a regulator gene tnaC located downstream of the promoter in a tryptophanase operon (tna), and (b) a DNA fragment containing a desired structural gene which can express by the promoter, which comprises cultivating the transformed microorganism in a culture medium while adding glucose as a carbon source continuously or intermittently so that the concentration of glucose is maintained within the range of 0.01 to 0.3 %, and thereby allowing the desired structural gene to express in the microorganism.

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CULTIVATION OF TRANSFORMED MICROORGANISMS

This invention relates to a process for cultivating a microorganism which comprises cultivating a microorganism transformed with a recombinant plasmid containing the desired structural gene derived from an animal, plant or microorganism which can express by a promoter in a tryptophanase operon, and thereby producing large amounts of the expression product of the desired structural gene, to novel recombinant plasmids which can be used in the process, and to microorganisms transformed with the plasmids.

There have recently been striking advances in the DNA recombinant technology of producing large amounts of useful substances by using host microorganisms holding recombinant plasmids having incorporated therein DNA fragments bearing the genetic information of useful substances derived from animals, plants or microorganisms. Production insulin or interferon using Escherichia coli as a host microorganism has already been under way.

In spite of the advances in the DNA recombinant technology, however, no process for producing large amounts of the desired useful substance using recombinant plasmids containing the desired structural gene has yet been established, and the development of a process for cultivating transformants efficiently is urgently in need.

On the other hand, the gene expression mechanism of a tryptophanase operon is controlled by an expression regulating mechanism, called "catabolite repression" (J. L. Bostford and R. D. DeMoss; J. Bsac., 1971, 105, 303 - 312). It is known that in a culture medium containing glucose as a main carbon source, the expression of the gene is very strongly inhibited.

Thus, although the promoter in the tryptophanase operon has the strong ability to express the desired structural gene, when it is desired to express the desired structural gene highly in a microorganism having a plasmid containing the promoter and the desired structural gene to be expressed by the promoter by cultivating the microorganism, a culture medium containing glucose as a main carbon source cannot be used, and one is compelled to use a culture medium containing other expensive carbon sources such as succenic acid. The inability of using a cheap and insustrially valuable glucose in cultivating a microorganism containing such a gene for the expression of a uesful structural gene by utilizing the strong ability to express the gene by the promoter in the tryptophanase operon constitutes a great setback in industrially utilizing the promoter.

The present inventors extensively studied the effective utilization of the strong gene expressing mechanism of the promoter in the tryptophanase operon (tna), and consequently found that by joining a DNA fragment (a) containing the structural gene to the downstream side of a DNA fragment (a) containing a promoter in the tryptophanase operon (tna) and a regulator gene tnaC located downstream of the promoter, not only the tryprophanase structural gene but also a foreign structural gene can be expressed strongly by the above promoter.

In spite of the fact that a microorganism transformed with a recombinant plasmid containing at least the DNA fragments (a) and (b) undergoes the above-mentioned catabolite repression (the inhibition of gene expression by glucose), if glucose is added continuously or intermittently so that its concentration is maintained at a specified low value in a culture medium containing L- or DL-tryptophan, the present inventors surprisingly found that the transformed microorganism does not undergo catabolie repression, and the desired gene was expressed highly, and the expression product can be obtained in large amounts.

As a method of cultivating a microorganism while glucose is added to the culture medium continuously or intermittently in some low concentrations, Japanese Patent Publication No. 42555/1986 already disclosed a method of cultivating a tryptophan synthase-producing Escherichia coli strain by adding glucose continuously or intermittently so that its concentration in the culture medium is maintaiend at 1 % or less.

It is known that the expression of a tryptophan synthase gene is controlled by a promoter in a tryptophan operon, and this gene expression mechanism does not undergo catabolite repression.

In fact, Agr. Biol. Chem., 38 (7), 1335 - 1334, 1974 states that when in the cultivation of the tryptophan synthase-producing Escherichia coli, the concentration of glucose in the culture medium is maintained at 0.5 and 1.8 %, the tryptophan synthase activity of the cultivated cells becomes 2.4 and 7.3 U/ml, showing that the enzyme activity increases as the concentration of glucose increases. Japanese Patent Application Publication No. 268175/1986 discloses that when in the cultivation of the tryptophan synthase-producing Escherichia coli, the concentration of glucose in the culture medium is maintained at 0.5 to 5 %, the tryptophan synthase activity is the highest.

The above-cited Japanese Patent Publication No. 42555/1986 discloses that when in the example, the concentration of glucose in the culture medium was maintained at 1.0 and 0.05 %, the amount of the cells

yielded increased to 2.69 times and the enzyme activity increased to 1.80 times for a glucose concentration of 1 %, and the amount of the cells yielded increased to 33.1 times and the enzyme activity increased to 2.15 times for a glucose concentration of 0.05 %, as compared with the case of cultivation in a culture medium containing 5 % of glucose at the start. From the results, at lower concentrations of glucose, both the amount of the cells yielded and the enzyme activity tended to show an increase. But the difference is only slight. There is no disclosure that the tryptophan synthase-producing strain used in the above cultivation method is a recombinant DNA in which the tryptophan synthase gene can be expressed by the promoter of the tryptophanase operon (tna). Furthermore, when the gene expression is inhibited by catabolite repression, only the expression of the gene is inhibited irrespective of the cell growth. As stated above, the Escherichia coli used in Japanese Patent Publication No. 4255/1986 is controlled by glucose both in the amount of the cells recovered and the enzyme activity.

It is considered from the phenomenon disclosed in Patent Publication No. 42555/1986 quite differs in mechanism from the catabolite repression but is a very specific phenomenon which the tryptophan synthase-producing strain Escherichia coli used in the method of the above patent document.

With regard to the cultivation of the tryptophan synthase-producing Escherichia coli, methods have previously been disclosed in which the cultivationm is carried out while maintaining glucose in the culture medium at various concentrations. However, to the best of the knowledge of the present inventors there has been disclosed no industrial process for cultivating Escherichia coli transformants by utilizing the gene expression mechanism of a promoter in tryptophanase operon (tna)

According to this invention, there is provided a process for cultivating a microorganism transformed with a recombinant plasmid at least containing (a) a DNA fragment containing a promoter in a tryptophanase operon (tna), and a regulator gene tnaC positioned downstream of the promoter and (b) a DNA fragment containing a desired structural gene which can express by the promoter, which comprises cultivating the transformed microorganism in a culture medium while adding glucose as a carbon source continuously or intermittently so that the concentration of glucose is maintained within the range of 0.01 to 0.3 % (wt/vol), and thereby allowing the desired structural gene to express in the microorganism.

The cultivation process in accordance with this invention will be described below in greater details.

I. Transformed microorganism

The microorganism to be cultivated by the process of this invention is a microorganism to be transformed with a recombinant plasmid containing

(a) a DNA fragment containing a promoter in a tryptophanase operon (tna) and a regulator gene tnaC positioned downstream of the promoter (to be sometimes abbreviated as "P fragment") and

(b) a DNA fragment containing a desired structural gene which can express by the promoter (to be sometimes abbreviated as "T fragment").

The transformed microorganism will be described below.

I-1. Recombinant plasmid

The recombinant plasmid to be introduced into the host microorganism in preparing the transformed microorganism at least contains the DNA fragment (a) (P fragment) and the DNA fragment (b) (T fragments). It can be stably held in the host microorganism and replicate. It may contain another DNA fragment.

The P fragment bears the function of expressing the desired structural gene which is desired to be expressed by being incorporated in the plasmid. A suitable source of its supply may be, for example, a chromosomal DNA of Escherichia coli for practical purposes. Advantageously used are chromosomal DNAs of E. coli ATCC 27325, E. coli ATCC 23282, E. coli ATCC 23437 and E. coli ATCC 23461.

A specific method of preparing the P fragment from the microorganism as a suppply source is described in Example 1, (1) and Example 2, (2). Its basic procedure will be described briefly. A chromosomal DNA containing a tryptophanase operon is extracted from a cultivation product of Escherichia coli, the supply source, by a conventional method [H. Saito and K. Miura: Biochemica Biophysica Acta 72, 619-629 (1963)]. The chromosomal DNA is digested with a suitable restriction endonuclease, and a DNA fragment containing a promoter in the tryptophanasse operon (tna) and a regulator gene tnaC. For example, if it is cut out with restriction endonucleases Bam HI and Hind III, a tryptophanase operon (tna DNA fragment having a molecular size of about 3.2 kb can be obtained. If this DNA fragment is further cut out

with restriction endonucleases Alu I and RsaI, a DNA fragment having a molecular size of about 630 bp and containing the tna promoter and the regulator gene tnaC can be prepared. This DNA fragment can be used as the P fragment.

If the desired structural gene in the T fragment is a tryptophanase structural gene (tnaA), the above tryptophanase (tna) DNA fragment having a molecular size of about 3.2 kb or an about 2.3 kb DNA fragment prepared by deleting about 900 bp from the BamHI site of the above DNA fragment with exonuclease III may be used as a DNA fragment in which the P fragment is linked to the T fragment.

On the other hand, the "desired structural gene" contained in the T fragment includes those structural genes derived from microorganisms, animals or plants encoding genetic information corresponding to proteins and peptides which can be expressed by the P fragment.

Typical examples of useful substances which such desired structural genes bear as genetic information include tryptophanase (E.C. 4. 1. 99.1), tryptophan synthase (E.C.4.2.1.20), chloramphenicol acetyltransferase (E.C.2.3.1.28), aspartase (E.C.4.3.1.1), threonine synthase (E.C.4.2.99.2S), homoserine kinase (E.C.2.7.1.39), various growth hormones, insulin, somatostatin, interferon and interleukin. DNA fragments (T fragment) containing the desired structural genes bearing the genetic information of these useful substances can be obtained from the cells of animals, plants or microorganisms by known methods. In the present invention, those derived from Escherichia coli are suitably used.

Specific preferred examples of T fragment are a DNA fragment containing the tryptophanase structural gene (tnaA) and a DNA fragment containing tryptophanase synthase structural genes (trpA, trpB). Specific examples of the sòuirce of the Escherichia coli K-12 strain are E. coli ATCC 27325, E. coli ATCC 23282, E. coli ATCC 2347 and E. coli ATCC 23461.

Examples of a DNA fragment containing the tryptophanase structural gene (tnaA) are a tryptophanase operon (tna)DNA fragment having a molecular size of about 3.2 kb obtained by digesting the above E. coli chromosomal DNA with restriction endonucleases Bam HI and Hind III, and an about 2.3 kb DNA fragment obtained by deleting about 900 bp from the Bam HI site of the above DNA. These DNA fragments result from linking the tryptophanase structural gene (tnaA) with the downstream side of the P fragment, and can be utilized as a DNA fragment containing both the P fragment and the T fragment at the same time.

An example of the DNA fragment containing tryptophan synthase structural genes (trpA, trpB) is a DNA fragment having a molecular size of about 2.6 kb and containing tryptophan synthase structural genes (trpA, trpB) obtained by infecting the above E. coli chromosomal DNA with phage ϕ (phi) 80, and then inducing it to prepare a large amount of phage taking up the tryptophan operon in the phage DNA, then extracting the phage DNA, digesting it with restriction endonuclease Bam HI to cut out a tryptophan operon DNA fragment having a molecular size of about 20 kb, and further digesting this DNA fragment with restriction endonuclease HindII.

Examples of other DNA fragments which the recombinant plasmid in accordance with this invention can contain include a DNA fragment containing a gene encoding the autonomous replicating ability of Col EI-type plasmids, a DNA fragments containing a gene encoding the plasmid partition system derived from F plasmid, and DNA fragments containing genes encoding the resistances of antibiotics (such as ampicillin, kanamycin and chloramphenicol).

One specific example of a preferred recombinant plasmid used in this invention is a plasmid holding

(a) a DNA fragment (P fragment) containing a promoter and a regulator gene (tna C) located downstream of the promoter in a tryptophanase operon (tna),

(b) a DNA fragment (T fragment) containing the desired structural gene that can be expressed by the promoter,

(c) a DNA fragment containing a gene encoding the autonomic replicating ability of a Col EI-type plasmid, and

(d) a KDNA fragment containing a gene encoding the plasmid partition system derived from an F factor plasmid.

The DNA fragment (c) used in combination with the P fragment and the T fragment, that is, "the DNA fragment containing a gene encoding the autonomic replication derived from a Col EI type plasmid" (to be abbreviated as "S fragment", includes DNA fragment containing a gene encoding the autonomic proliferation of a Col EI-type plasmid having a copy number of 20 to 30 per chromosome. Typical examples of this S fragment are an S fragment having a molecular size of about 4.3 kb and being derived from plasmid pBR322, an S fragment having a molecular size of about 5.1 kb and being derived from plasmid pkk232-8, and an S fragment derived from plasmid pBR325.

The P fragment, the T fragment and the S fragment are further combined with the DNA fragment (d) containing a gene encoding the plasmid partition system derived from an F plasmid. The F plasmid is a known plasmid having a molecular size of 94.5 kb (62 x 10^6 daltons) having a structure described, for

example, in the gene map of Figure 1 at page 98 of "Protein, Nucleic acid and EnzymesS", vol. 27, No. 1 (1982) and the physical map by EcoRI. It exists with a copy number of 1 -2 per chromosomal cell in enteric bacteria. This plasmid has such a mechanism that after the cell division. This plasmid has a mechanism whereby it is accurately partitioned to daugher cells after cell division (the proliferation of a host in spite of a low copy number level as in this case is called the "stringent" control of replication. It has already been found that such a function of stringently controlling proliferation in the F-plasmid is carried out on a fragment, called mini-F, which has a molecular size of 9.2 kb and can replicate autonomously [see "Molecular & General Gentics", 196, 185 - 193 (1984)]. It is also known that the mini-F can be cut out from the P fragment with restriction endonuclease EcoRI.

The plasmid in accordance with this invention utilizer the plasmid partition system encoded by the mini-F. Thus, the "DNA fragment containing genes which encodes the plasmid partition system and are derived from the F fragment. This DNA fragment means a DNA fragment which encodes the mechanism for accurately partitioning the F plasmid to daughter cells. A typical example is a mini-F fragment having a molecular size of about 9.2 kb which can be cut out using restriction endonuclease EcoRI. The plasmid that can be used in this invention may contain another DNA fragment bearing another genetic information, such as a DNA fragment containing an ampicillin-resistance gene or a kanamycin-resistance gene as an antibiotic resistance marker so long as it essentially contains the above described P fragment, T fragment, S fragment and F fragment.

Construction of the recombinant plasmid from these DNA fragments may be carried out by known methods as described in a standard testbook on gene manipulation technology T. Maniatis, E. F. Fritsch and J. Sambrook, "Molecular Cloning", Cold Spring Harbor Laboratory (1982)].

A typical example of the recombinant plasmid that can be used in this invention consists substantially of four DNA fragments, i.e., the P fragments, T fragment, S fragments and T fragment. More specifc examples include

(1) a plasmid having a molecular size of about 10.2 megadaltons about 15.5 kb) in which the T fragment is a DNA fragment containing th e tryptophanase structural gene (tna A). This plasmid is named "plasmid pSMTP4" by the present inventor (see Example 1);

(2) plasmids pMTP1 and pMTP1R having a molecular size of about 10.7 megadaltons (about 15.4 kb) in which the T fragment is a DNA fragment containing (these plasmids and thier method of preparation are disclosed in Japanese Laid-Open Patent Publications No. 28393/1988, and the description of this patent document is cited herein in lieu of describing them in detail);

(3) A plasmid having a molecular size of about 9.9 megadaltons (about 16.6 kb) in which the T fragment is a DNA fragment containing the tryptophan synthase structural gene (trpB, trpA). The present inventors named it "plasmid pMTY20" (see Example 2 given hereinafter. Of these, plasmid pdSMTP4 and plasmid pMTY20 are preferred. The plasmids pMTP4 and pMT20 are novel plasmids not described in the prior literature, and constitute part of the present invention.

The plasmid pMTP4 has the following recognition sites of restriction enconucleases and the length of fragments.

Plasmid pMTP4		
Restriction endonuclease	Number of recognition sites	Length of fragments (kb)
EcoRI	2	9.2, 6.3
BamHI	3	2.4, 10.7, 2.4
HindIII	2	14.7, 0.8

The pMTP4 having the above characteristics can be produced, for example, by the following procedure.

First, the DNA fragment (P + T) in which the tryptophanase promoter, the regulator gene tnaC positioned downstream of the promoter and the tryptophanase located gene tnaA are jointed is prepared, for example, by extracting a chromosomal DNA from, for example, E. coli having a tryptophanase operon in the chromosomal gene, for example Escherichia coli K-12 strain (ATCC 23282, ATCC 23437, ATCC 27325 or ATCC 23461 and cutting out a tryptophanase operon DNA fragment using restriction endocnucleases BamHI and HindIII by a conventional method [E. F. Fritsch, Sambrook, "Molecular Cloning", p. 164 -165, Cold Spring Harbor Laboratory] to obtain a DNA fragment having a molecular size of about 3.2 kb, and the 900 bp is deleted from the BamHI site of the fragment to obtain (P + T) fragment having a size of about

2.3 kb and containing the tryprophanase promoter, the regulator gene tnaC located downstream of the promoter, and the tryptophanase structural gene (tnaA).

On the other hand, the mini-F fragment can be prepared by taking out a microorganism holding the F plasmid such as E. coli K-12 strain (ATCC 15153, ATCC e23589 or ATCC e23590), for example by a known method, for example, P. Guerry, D. L. La Blanc, S. Falkow: J. Bact., 116, 1064 (1973) by cutting out the mini-F fragment having a molecular size of about 9.2 kb from the F plasmid using restriction endonuclease EcoRI.

On the other hand, it is convenient to use plasmid pBR322 as a typical example of ColEI plasmid as a supply source of the DNA fragment containing a gene encoding the autonomous replication of Col EI plasmid.

The prepared (P + T) fragment is combined with the plasmid pBR322 treated with restriction endonucleases HamHI and Hind III and T4 DNA ligase was caused to act on them to prepare a plasmid resulting from incorporating the (P + T) fragment in the plasmid pBR322. Then, the resulting plasmid was cleaved with restriction endonuclease EcoRI and is combined with the mini-F fragment prepared as above. The action of T4 DNA ligase on them gives the desired plasmid pMTP4.

The specific method of preparing the plasmid pMTP4 will be described in detail in Example 1 below.

On the other hand, the plasmid pMTY20 shows the following restriction endonuclease recognition sites and the lengths (kb) of the fragments with the following endonucleases.

Plasmid pMTY20		
Restriction endonuclease	Number of recognition sites	Length of the fragments (kb)
EcoRI	2	9.2, 7.4
HindIII	3	9.3, 5.0, 2.3
SalI	4	12.8, 2.4, 0.8, 0.6
BamHI	5	6.8, 4.0, 2.7, 2.4, 0.7

The plasmid pMTY20 having the above characteristics can be produced, for example, by the following procedure.

The DNA fragment (containing the tryptophanase promoter and the regulator gene (tnaC) located downstream of the promoter may be prepared by extracting a chromosomal DNA from E. coli having a tryptophanase operon, such as E. coli K-12 strain (ATCC 23282, ATCC 23437, ATCC 27325, or ATCC 23461), and cutting out a tryptophanase operon DNA fragment from the chromosomal DNA in a customary manner [see E. F. Fritsch, Sambrook, "Molecular Cloning" (1982), pp. 164 - 165, Cold Spring Harbor Laboratory] using restriction endonucleases Bam HI and Hind III. Further by using restriction endonucleases R sal and AluI, an about 630 bp DNA fragment containing the tryptophanase promotoer and the regulator gene tnaC located downstream of the promoter. Furthermore, by imparting = BamHI linker, a P fragment is obtained.

The T fragment (a DNA fragment containing a tryprophan synthase structural gene may be prepared by infecting a E. coli, such as E. coli K-12 strain (ATCC 27325, ATCC 23282, ATCC 23437, ATCC 23437 or ATCC 23461 with a phage, such as phage ϕ (phi) 80 (ATCC 11456a-B1) and by the lysis and induction phenomena, a large amount of a phage taking up a tryprophan operon in the phage DNA was prepared [R. M. Denney, C. Yanofsky; J. Bacteriol., 118, 505 (1974). From the phage, the phage D is extracted by a customary method (E. F. Fritsch, Sambrook: "Molecular Cloning" (1982, pp. 164 - 165, Cold Spring Harbor Laboratory). By using restriction endonucleases such as BamHI, EcoRI, a tryptophan operon DNA fragment is cut out. This DNA fragment is further partially digested with restriction endonuclease HincII to give a DNA fragment containing trpA and trpB genes.

Then, the resulting DNA fragment and a SalI linkder are mixed, and ligated by a T4 DNA ligase to give a T fragment having a Sal 1 site at both ends of the trpA/trpB fragment.

On the other hand, the mini-F fragment (T fragment) is prepared by taking out the F plasmid from a microorganism holding the F plasmid, such as E. coli K-12 (ATCC 15153, ATCC e2389 or ATCC c23590), by a known method described, for example, in P. Guerry, DL. Le Blanc, S. Falkow, J. Bact., 116, 1064, and cutting out an about 9.2 kb mini-F fragment using restriction endonuclease EcoRI from the F plasmid.

On the other hand, it is convenient to use plasmid pBR322 typical of a Col EI plasmid as a source of supply of the DNA fragment (S fragment) containing a gene encoding the autonomous replication of the Col

EI plasmid.

A fragment containing a tryptophanase promoter, a regulator gene tnaC and a tryptophan synthase structural gene (trpA, trpB) is obtained by inserting the P fragment and the T fragment obtained as above into the BamHI site and the SalI site of plasmid pUC119, and again partially digesting the resulting DNA fragment with EcoRI and Hind III. The resulting DNA fragment is inserted into the EcoRI and HindIII sites of the plasmid pBR322 (S fragment) to give the desired plasmid pMTY20.

A specific method of preparing the plasmid pMTY20 will be shown in detail in Example 2 below.

I-2. Host Microorganism

Host microorganisms that can be transformed with the recombinant plasmids described in section I-1 are suitably microorganisms belonging to Escherichia coli. Depending upon the type of the plasmid, such microorganisms as Pseudomonas putida, Brevibacterium flavum and Bacillus subtilis may also be used as hosts.

Examples of the microorganisms belonging to Escherichia coli are E. coli K-12 strains, ATCC 27325, ATCC 23282, ATCC 23437 and ATCC 23451

I-3. Transformation

The host microorganisms described in I-2 may be introduced into the recombinant plasmids described in section I-1 by methods known per se, for example M. Mandel, A. Higa: SJ. Mol. Biol., 53, 159 (1970).

Specific examples of microorganisms transformed as above are shown below.

(1) Escherichia coli YK3007 (FERM BP-2803) holding the plasmid pMTP4

(2) Escherichia coli K-12 YK3002 (FERM BP-1733) holding the plasmid pMTP1, and Escherichia coli K-12 YK 3003 holding the plasmid pMTP1R (FERM BP-1734)

(3) Escherichia coli YK2017 (FERM BP-2804) holding the plasmid pMTY20

The transformed Escherichia coli strain are deposited under the above parenthesized FERM numbers at the Fermentation Research Institute, Agency of Industrial Science and Technology, 1-3, Higashi 1-chome, Tsukuba-shi Ibaraki-ken 305, Japan under the Butapest Treaty on the International Recognition of the Deposit of Microorganisms for the purposes of Patent Procedure.

II. Cultivation

The transformed microorganism is cultivated in accordance with the process of this invention in a culture medium containing L- or DL-tryptophan while continuously or intermittently adding glucose as a carbon source to the culture medium so that the concentration of glucose is maintained at 0.01 to 0.3 % (wt/vol). As a result, the transformed microorganism does not undergo catabolite repression, but the gene expression mechanism of the promoter in the tryptophanase operon (tna) acts strongly. Thus, the desired structural gene expresses to produce the useful substance in a high yield.

The concentration of glucose in the medium may be maintained during the cultivation period at 0.01 to 0.3 % (wt/vol), preferably 0.02 to 0.25 % (wt/vol), more preferably 0.03 to 0.2 % (wt/vol). This enables the use of other industrially expensive carbon sources to be omitted. If desired, other carbon sources such as glycerol, succinic acid, acetic acid, fumaric acid and malic acid may be used together.

Furthermore, L- or DL-tryptophan is also added to the culture medium used in this invention. This enables the gene expression mechanism of the promoter in the tryptophanase operon (tna) to act strongly.

The concentration of L- or DL-tryptophan in the culture medium is not strictly limited. Preferably it is 0.1 to 2 % (wt/vol), more preferably 0.05 to 0.5 % (wt/vol). The time of addition of L- or DL-tryptophan may be in an early stage or in an intermediate stage of cultivation, preferably before, the cultivation reaches the later stage of logarithmic growth.

Further, nutrients such as nitrogen sources, inorganic salts and growth promoting substances are properly added to the culture medium used in this invention depending upon the type of the microorganism. Nitrogen sources to be included in the culture medium include, for example, ammonium salts such as ammonium sulfate, ammonium chloride, ammonium nitrate and ammonium phosphate, nitric acid salts such as potassium nitrate, sodium nitrate and ammonium nitrate; organic nitrogen such as glutamic acid, glutamine, aspartic acid and asparagine; and ammonia, to be used either alone or in mixture. Examples of

the inorganic salts are potassium monohydrogen pbosphate, potassium dihydrogen phosphate, magnesium sulfate, iron sulfate and manganese sulfate either alone or in combination. There is no particular restriction on the growth promoter substances, and examples that can be cited are vitamins such as thiamin and biotin, amino acids such as methionine and cystein, and substances wholly or partly containing vitamins or amino acids, such as yeast extract, polypeptone, meat extract, corn steep liquor and Casamino acid.

There is no limitation on the contents of these nutrients in the culture medium, and may be those contents which are used in ordinary cultivations. The nutrients derived from natural materials are suitably contained in amounts of usually 1 to 0.1 %, preferably 0.5 to 0.2 %, in the culture medium.

The cultivation of the microorganism in the culture medium having the above composition may be carried out under aerobic conditions by using the same cultivation device as used normally. By passing air or another oxygen-containing gas, through the culture medium and/or stirring the culture medium, the concentration of the dissolved oxygen in the culture medium may be maintained generally at 1 to 8 ppm, preferably at 2 to 7 ppm, and the cultivation can be performed more effectively. = The addition of glucose to the culture broth may be carried out continuously, or intermittently at a certain time interval or while monitoring the concentration of glucose.

The "concentration of dissolved oxygen" may be measured, for example, by using the glucose A Test Wako (a product of Wako Pure Chemicals, Co., Ltd.) or a glucose analyzer GLU-1 (a product of Toa Denpa Co., Ltd.), and the "concentration of dissolved oxygen" may be measured by a DO converter (a product of Oriental Electric Co., Ltd.).

The cultivation conditions may be selected so as to be suitable for the type of the transformed microorganism. Generally, the cultivation temperature is about 10 to about 45 °C, preferably about 25 to about 40 °C, and the pH of the culture medium may be maintained at about 3 to 10, preferably about 5 to 9. Where the pH of the culture medium varies during the cultivation, alkalies such as ammonia, sodium hydroxide, potassium hydroxide, sodium carbonate and sodium hydrogen carbonate, or acids such as sulfuric acid and hydrochloric acid are desirably added to adjust the pH. The cultivation time may be adjusted usually to about 5 to 48 hours.

By performing the cultivation as described above the structural gene bearing the useful substance and be accumulated in very large amounts in the microbial cells or the culture broth. The useful substance can be recovered from the microbial cells or the culture broth by known methods such as centrigufal separation and ultra-filtration.

The following examples illustrate the present invention more specifically. It should be understood that these examples are given as an aid to a understanding of the present invention, and do not limit the scope of the invention in any way.

The drawings which are referrred to in these examples are as follows:

Fig. 1 is a restriction endonuclease cleavage map of plasmid pMTP4.

Fig. 2 is a restriction endonuclease map of plasmid pMTY20.

Fig. 3 is a restriction endonuclease cleavage map of plasmid pCAT-1.

In these examples, (1) the number of recognition sites of restriction endonucleases, and the sizes of the fragments, (2) the tryptophanase activity, (3) the tryptophan synthase activity, (4) chloramphenicol acetyltransferase (CAT) activity, (5) the amounts of proteins and glucose in a sample enzyme solution and (6) the amounts of the cells harvested and (7) dissolved oxygen concentration were measured by the following methods

(1) Number of recognition sites by restriction endonuclease and the size of a fragment:-

The "number of recognition sites" by a restriction endonuclease can be determined from the number of separable fragments which is examined by completely digesting the DNA fragment or plasmid with an excess of a restriction endonuclease and subjecting the digestion product to agarose gel electrophoresis and polyacrylamide gel electrophoresis.

The "size of a fragment" and the sizes of the individual DNA fragments of a plasmid are calculated on the basis of a standard line drawn by migration distances on the same agarose gel of a DNA fragment of a known molecular size obtained by digesting DNA of ϕ (phi) phage of Escherichia coli with restriction endonuclease HindIII when agarose gel electrophoresis is used; and on the basis of a standard line drawn by migration distances on the same polyacrylamide gel of a DNA fragment of a known molecular size obtained by digesting DNA of ϕ (phi) phage of Escherichia coli with restriction endonuclease HaeIII. The size of the plasmid is calculated by adding the sizes of the individual DNA fragments, fragments of at least 1 kb is deremined by using the results obtained by 1 % agarose gel electrophoresis, and fragments of

about 0.1 kb to less than 1 kb, by using the results obtained by a 4 % polyacrylamide gel electrophoresis.

(2) Tryptophanase activity

Cells centrifuged (4000 rpm, 15 minutes, 4 °C) from 20 ml of the culture broth, were suspended in 20 ml of a 100 millimole phosphase buffer (pH 8.0, and then again centrifuged (4000 rpm 15 minutes, 4 °C). The cells harvested were suspended in 2 ml of the same buffer as above, and disrupted by ultrasonication (Sonifier 200 made by Branson). The treated product was centrifuged (12000 rpm, 40 minutes, 4 °C), and the supernatant was used as a sample enzyme solution.

The above enzyme solution was diluted with a proper amount of 100 millimole phosphate buffer (pH 8.0) and 0.1 ml of the diluted solution was added a test tube containing 0.9 ml of a reaction solution [a 100 millimole phosphate buffer (pH 8.0) containing 10 millimoles of L-tryptophan and 0.04 millimoles of pyridoxalphosphate]. At 37 °C the mixture was subjected to reaction under shaking in a constant temperature water vessel for 20 minutes. The reaction was stopped by adding 1 ml of 10 (wt/vol) trichloroacetic acid, and the resulting indole in the supernatant was quantitatively determined by gas chromatography CC-3BF, made by Shimazu Seisakusho). The tryptophanase activity was calculated as the amount of indole formed per hour per unit amount of protein, and was expressed as a relative value by taking the activity in a comparative example as 1.

(3) Tryptophan synthase activity.

Cells centrifuged (4000 rpm, 15 minutes, 4 °C) from 20 ml of the culture broth was suspended in 20 ml of 100 mM Tris-HCl buffer (pH 7.8), and again centrifuged (4000 rpm, 15 minutes, 4 °C). The cells were harvested and suspended in 2 ml of the above buffer and disrupted by ultrasonication (Sonifier 200, made by Branson). The treated product was centrifuged (12000 rpm, 40 minutes, 4 °C). The resulting supernatant was used as a sample enzyme solution.

The enzyme activity of the sample solution was carried out in accordance with the method of Yanofsky et al. [Method in Enzymology, vol. 5, 794 (1962)].

The tryptophan synthase activity was calculated as the amount of indole which formed per hour per unit amount of protein, and was expressed as a relative value when the activity in a comparative example was taken as 1.

(4) Activity of chloramphenicol acetyl transferase (CAT) activity

The culture broth was centrifuged, and the cells were harvested. The cells were washed with 50 ml of 10 mM Tris buffer (pH 7.8). The washed cells were again centrifuged, and the cells were harvested to obtain 200 mg of wet cells. The harvested cells were suspended in 1 ml of a 10mM Tris buffer (pH 7.8), and ultrasonicated. The enzyme activity was measrued in a customary manner by the method of W. V. Shaw et al. [Method in Enzymology, Academic Press, New York (1975), vol 43, p. 737] after the resulting cell debris was properly diluted with a 10 mM Tris buffer. The amount of chloramphenicol acetyl transferase activity was calculated as the amount of chloramphenicol acetylated per minute per unit amount of protein, and expressed as a relative value when the activity in a comparative example was taken as 1.

(5) The amount of protein in the sample enzyme solution was measured by the method of Lowry et al. (Journal of Biological Chemistry, vol 193, p. 265, 1951), and the amount of glucose in the enzyme solution, by the glucose C test Wako (made by Wako Pure Chemicals Co., Ltd.).

(6) Amount of cells harvested

The amount of wet cells recovered after centrifuging (8000 rpm, 20 minutes 4 °C) 50 ml of the culture broth.

(7) Dissolved oxygen concentration

The dissolved oxygen concentration was measured by a DO converter (made by Oriental Electric Co., Ltd.)

EXAMPLE 1

Preparation of plasmid pMTP4 and transformation of Escherichia coli K-12 strain with plasmid pMTP4

(1) Preparation of a DNA fragment containing a promoter in the tryptophanase operon (tna), a regulator gene tnaC located downstream of the promoter and a tryptophanase structural gene (tnaA):-

100 ml of an L-medium (10 g of trypton, 5 g of yeast extract, 1 g of glucose, 5 g of NaCl, 1 liter of distilled water; pH 7.2) was put in a 500 ml Erlenmeyer flask, and sterilized at 120 °C for 15 minutes.

Escherichia coli K-12 (ATCC 27325) was inoculated in this culture medium, and cultivated at 37 °C for 15 hours. Then, 2 ml of of the culture broth was taken, and then inoculated anew in 100 ml of the culture medium and again cultivated at 37 °C for 4 hours.

After the cultivation, the entire cultivation liquid was centrifuged (8000Xg, 15 minutes, 4 °C), and the cells were harvested. The cells were then suspended in 50 ml of a solution containing 50 mM Tris buffer (pH 8.0) and 10 mM EDTA.2Na solution. Then, lysozyme was added so that its final concentration reached 2 mg/ml. After standing for 5 minutes, 6 ml of a 10 % solution of sodium dodecyul sulfate was added, and the mixture was kept warm at 65 °C for 30 minutes. After this lysis, 15 ml of a 5M solution of NaCl was added, and the mixture was cooled at 0 °C for 1 hour. The entire amount of the mixture was centrifuged (12000 x g, 60 minutes, 4 °C), to collect the supernatant fraction, and ethanol was added in twice its amount. After mixing, the mixture was centrifuged (5000 x g, 10 minutes, 4 °C). The resulting precipitate was dissolved in 10 mM Tris buffer (pH 7.5)-1 mM EDTA.2Na solution, and the solution was treated with phenol (the protein removing treatment) and treated with decomposing enzyme to give 1.5 mg of DNA finally.

The so prepared chromosomal DNA (25 micrograms) was digested with 5 units each of restriction endonucleases HindIII and BamHI at 30 °C for 1 hour to prepare solution of Hind III and BamHI digestion products of the chromosomal DNA. pBR322 (1 microgram) was digested with 1 unit each of restriction endonucleases HindIII and BamHI at 30 °C for 1 hour. The resulting digestion product solution was mixed with the above decomposition product solution. The mixture was reacted with a 50 mM Tris buffer (pH 7.6), 10 mM dithiothreitol, 1 mM ATP, 10 mM $MgCl_2$ and 1 unit of T4 ligase (the concentrations of these components were the final concentrations) at 16 °C for 15 hours. By using the resulting solution, Escherichia coli K-12 strain (tryptophanase-deficient mutant and a tryptophan-requiring mutant) was transformed by a conventional method [M. Mandel, A. Higa: J. Mol. Biol., 53, 159 (1970)], and then coated on a selective medium consisting of 7 g of K_2HPO_4, 2 g of KH_2PO_4, 1 g of $(NH_4)_2SO_4$, 0.1 g of $MgSO_4.7H_2O$, 5 g of Casamino acid, 10 mg of indole, 50 mg of adenine, 2g of succinic acid, 20 g of agar, and 1 lliter of deionized water. The cells which grew on the selective medium were inoculated in an L-medium to which ampicillin was added in a final concentration of 50 micrograms/ml, and cultivated at 37 °C for 7 hours. The cells were harvested by centrifugation (8000Xg, 10 minutes, 4 °C). By using these cells, the tryptophanase activity was examined.

50 mg of wet cells were added to 1 ml of a reaction solution for measurement of the tryoptophanase activity (0.1 M phosphate buffer pH 8.0, 10 mM tryptophanase, and reacted at 37 °C for 30 minutes. The indole was colored with p-dimethylbenzaldehyde to give a red colored cell. From the cells, a plasmid was extracted by the alkali SDS method. [Maniatis, E. F. Fritsch and J. Sambrook: "Melecular Cloning" (1982) pages 90 to 91 and digested with BamHI and HindIII. The molecular sizes of the product examined by using an agarose gel. It was found that a DNA fragment having a molecular size of about 3.2 kb was seen to be inserted into the HindIII and BamHI sites of pBR322 (this plasmid will be referred to as pBR322tna).

The resulting transformants were cultivated in a liquid medium, and plasmid pBR322tna was separated from the culture liquid and purified. The plasmid DNA was completely digested with restriction endonucleases BamHI and HindIII at 37 °C for 1 hour. The digestion product was separated by 0.8% agarose gel electrophoresis, and from the gel, a DNA fragment fraction containing about 3.2 kb tryptophanase was cut out. A DNA was extracted, and purified to obtain a DNA fragment. This DNA fragment was then introduced into plasmid pUC119, and pUC119tna was prepared as follows.

The plasmid pUC119 is a plasmid having a molecular size of about 3.1 kb which can replicate in Escherichia coli and expresses ampicilin resistance. It can be purchased from Takara Shuzo Co., Ltd., Japan.

The plasmid pUC119 (0.9 micrograms) was reacted with restriction endonucleases BamHI and HindIII at 37 °C for 1 hour to digest it completely. The digestion product was mixed with a BamHI and HindIII DNA fragment containing the tryprophanase structural gene (tnaA). To inactivate the restriction endonucleases, the mixture was heat-treated at 65 °C for 10 minutes. As final concentrations, 50 mM Tris buffer (pH 7.6), 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP and 1 unit of T4 DNA ligase were added to the deactivation solution, and the solution were maintained at 16 °C for 15 hours. Escherichia coli JM109 competent cells (a product of Takara Shuzo Co., Ltd.) were transformed by using the resulting solution.

The transformants were cultivated for 24 hours in L medium (10 g of Trypton, 5 g of yeast extract, 5 g of NaCl, 1 g of deionized water, pH 7.2) containing 50 micrograms/ml (final concentratio) of ampicillin, 100 micrograms/ml (final concentration) of IPTG (isopropyl-beta-D-thiogalactopyranoside), and 100 micrograms/ml (final concentration of X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside, and grown transformants were obtained. Among them, those which grew in white colonies were selected, and the plasmids were esxtracted by the alkali-SDS method [T. Maniatis, E. P. Fritsch, J. Sambrook, "Molecular Cloning" (1982) pages 90 to 91 (the resulting plasmid will be referred to as pUC119tna).

The plasmid pUC119tna DNA was completely digested with restriction endonucleases BamHI and BbeI at 37 °C for 1 hour. From this 3.2 kb fragment, an about 900 bp region from the BamHI site was deleted with exonuclease III (a product of Takara Shuzo Co., Ltd.), and the Bbe I site was made a blunt end by treatment with S_1 nuclease (a product of Takara Shuzo, Co., Ltd.). Then, the fragment was mixed with a Bam HI linker (a product of Takara Shuzo Co., Ltd.), and 50 mM Tris buffer (pH 7.6), 10 mM of $MgCl_2$and 1 unit of T4 DNA ligase were added (these concentrations were final concentrations), and the reaction was carried out at 16 °C for 15 hours to perform ligation and reconstruct the plasmid.

Escherichia coli K-12 strain (tryptophanase-deficient and tryptophan-requiring strain) was transformed using the resulting plasmid solution in a customary manner [M. Mandel, A. Higa: J. Mol. Biol., 53 159 (1970)], and coated on a selective medium [7 g of K_2HPO_4, 2 g of KH_2PO_4, 1 g of $(NH_4)_2SO_4$, 0.1 g of $MgSO_4.7_2O$, 5 g of Casamino acid, 10 mg of indole, 50 mg of adenine, 2 g of glucose, 20 g of agar, and 1 liter of deionized water].

The grown cells on the culture medium were cultivated in a liquid culture medium in accordance with a conventional method, and ohe plasmid DNA was extracted from the culture broth. The plasmid DNA was completely digested with restriction endonucleases BamHI and HindIII at 37 °C, and separated by 0.8 % agarose gel electrophoresis to select a gel having about 2.9 kb pUC118 portion and a gel showing a size of about 2.3 kg (tna portion). This about 2.3 kb DNA fragment was cut out from the gel, and the DNA was extracted and purified to prepare an about 2.3 kb fragment containing the tryprophanase promoter, the regulator gene tnaC and the tryprophanase structural gene (tnaA).

Then, the plasmid pkBR322 DNA was completely digested with restriction endonucleases BamHI and HindIII at 37 °C for 1 hour, and the digestion product was mixed with the about 2.3 kb fragment. The mixture was heat-treated at 65 °C for 10 minutes to inactivate the restriction endonucleases. Then, 50 mM Tris buffer (pH 7.6), 10 mM dithiothreitol, 1 mM ATP, 10 mM $MgCl_2$ and 1 unit of T4 DNA ligase were added (the concentrations were final concentrations), and reacted at 16 °C for 15 hours to perform ligation. Escherichia coli HB101 competent cells (a product of Takara Shuzo Co., Ltd.) were transformed by using the resulting solution. The resulting transformants were cultivated in a culture medium, and the plasmid DNA was extracted from the culture broth. The plasmid DNA was analyzed by using restriction endonucleases BamHI and HindIII. A 2.3 kb DNA was seen to be inserted into the BamHI and HindIII sites of pBR322 (this plasmid will be referred to as pBR322tnal).

(2) Preparation of a mini-F fragment

Escherichia coli K-12 strain (ATCC 15153) was inoculated in one liter of L medium (Trypton 10 g, yeast extract 5 g, NaCl 5 g, glucose 1 g, water 1 liter; pH 7.2), and cultivated with shaking at about 37 °C for 4 hours. The cells were harvested, treated with lysozyme, and lyzed with sodium dodecylsulfate (SDS). The lysate was centrifuged for 40 minutes at 32,000 x g. The supernatant was separated, subjected to cesium chlorideethidium bromide euilibrium density gradient centrifugation and then dialyzed to separate a solution containing the F plasmid. The solution was precipitated with ethanol to finally collect about 20 micrograms of the F plasmid.

The, 5 micrograms of the F plasmid was taken and subjected to the action of restriction endonuclease

EcoRI for 1 hour at 37 °C to cleave the DNA. As a result, a DNA solution containing a mini-F fragment having a size of about 9.2 kb was prepared.

(3) Preparation of the desired plasmid

The plasmid pBR322tnaI DNA obtained in section (1) above was completely digested with restriction endonuclease EcoRI at 37 °C for 1 hour. The digestion product was mixed with the mini-F fragment obtained in section (2). Teh mixture was heat-treated for 10 minutes at 65 °C to inactivate the restriction endonuclease. Then, 50 mM Tris buffer pH 7.6, 10 mM $MgCl_2$, 1 mM ATP and 1 unit of T4 DNA ligase were added (the concentrations were final concentrations, and the mixture was kept cool at 16 °C for 15 hours to perform ligation (this prosmid will be provisionally called pBR322Ftna).

(4) Transformation of Escherichia coli K-12 strain by plasmid pMTP4

Using the plasmid DNA prepared in section (3), Escherichia coli K-12 strain (ATCC 27325) was transformed in a customary manner to obtain transformants. They were cultivated in a liquid culture medium, and the plasmid pBR322Ftna was separated and purified, and named plasmid pMTP4.

This plasmid pMTP4 was digested with various restriction endonucelases, and the molecular sizes of the fragments were measured. The results are shown in Table 1.

Table 1

Restriction endonuclease	Number of recognition sites	Molecular weight (megadaltons; kb in the parentheses)
EcoRI	2	6.1 (9.2), 4.2 (6.3)
BamHI	3	1.6 (2.4), 7.1 (10.7) 1.6 (2.4)
HindIII	2	9.8 (14.7), 0.5 (0.8)

The restriction endonuclease cleavage map of the plasmid pMTP4 is shown in Fig. 1.

Escherichia coli YK3007 transformed with the plasmid pMTP4 was deposited on February 16, 1989 at Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, 1-1-3, Higashi, Tsukubashi, Ibaraki-ken, Japan under FERM P-10543 (transferred to international deposition FERM BP-2803 on March 14, 1990 under the Budapest Treaty).

(5) Stability of transformed E. coli YK3007

100 ml of the above L medium containing 2 g/liter of glucose as a carbon source was put in a 500 ml Erlenmeyer flask, and sterilized at 120 °C for 15 minutes. The transformants obtained in Example 1 were inoculated in the sterilized culture medium obtained above, and cultivated under shaking at 37 °C for 24 hours. Then, 100 ml of the culture medium prepared as above and put in a 500 ml Erlenmeyer flask and sterilized at 20 °C for 15 minutes so that the number of cells per ml was 50. Likewise, they were cultivated at 37 °C for 24 hours under shaking.

Then the cells were harvested by centrifugation, and washed. They were coated on a fixed amount on an L-medium to which ampicillin was added at a rate of 50 micrograms/ml and on a plate medium prepared as L-medium without addition of any additional component, and cultivated for one day at 37 °C. The grown colonies were counted.

As a result, it was found that the number of colonies grown on the ampicillin-containing L medium was the same as thse grown on the L medium to which no additional component was added. It was also determined that the colonies grown on the L-medium were all grew on a minimum medium (containing 10 mg/l of indole)containing no tryptophan. In other words, the stability of the plasmid was very high.

EXAMPLE 2

Preparation fo plasmid pMTY20 and transformation of Escherichia coli SK-12 strain with the plasmid pMTY20

(1) Preparation of a DNA fragment containing tryptophan synthase structural genes (trp A, trp B)

(A) Preparation of phage ϕ (phi) 80pt

Escherichia coli K-12 strain (ATCC 27325) was inoculated in 100 ml of L medium (having the same composition as indicated above), and cultivated with shaking at 37 °C for 4 hours. 0.5 mg of the culture broth was mixed with 0.1 ml of an aqueous solution of phage ϕ (phi) 80 (ATCC 11456a-B_1) (10^5/ml) in L medium soft agar (L medium + agar powder), and the mixture was overlaid on an L medium agar plate. When the plate was cultivated at 37 °Cfor about 5 hours, a plaque formed. When the cultivation was continued further for 2 to 3 days at 37 °C, grown colonies of phage ϕ (phi) 80 lysogenic bacteria formed in the plaque. The lysogenic bacteria were cultivated in L medium at 37 °C for 4 hours and plated on the same L medium agar plate by ultraviolet radiation (400-800 ergs/mm^2, 10-20 seconds), phage ϕ (phi) 80 pt (a phage DNA containing tryptopha operon) was prepared.

(B) Preparation of a tryptophan operon DNA fragment

Escherichia coli K-12 strain (ATCC 27325) was inoculated in 1 liter of L medium having the same composition as indicated above, and cultivated with shaking at about 37 °C for about 3 hours. In the logarithmic growth period, 10 ml of a 25 % (w/v) glucose solution and the phag 80 pt solution prepared in (A) were added (moi 20) in a concentration of 10^{11}/ml. The shaking culture was continued for 5 hours, and by the addition of chloroform, a large amount of phage ϕ (phi) 80 pt was prepared [see T. Maniatis, E. F. Fritsch, J. Sambrook: "Molecular Cloning" (1982), pages 76-80, Cold Spring Laboratory].

The phage ϕ (phi) 80 pt solution obtained was dialyzed against Tris buffer (pH 7.8), and by the DNA extracting method using phenol (see page 85 of the above-cited "Molecular Cloning"), the phage DNA was recovered and purified. The phage DNA was reacted with restriction endonuclease BamHI at 30 °C for 30 minutes to obtain a tryptophan operon DNA fragment (size: about 20 kb).

(C) Preparation of plasmid pBR322trp

The tryptophan operon fragment obtained in (B) was reacted with restriction endonuclease SalI and XhoI at 37 °C for 1 hour to cut out a DNA fragment having a molecular size of about 7.4 kb. Then, the reaction solution was heat-treated at 65 °C for 5 minutes to deactivate the restriction endonucleases. It was then mixed with plasmid pBR322 treated with restriction endonuclease SalI. Then, 50 mM Tris buffer (pH 7.6), 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP and 1 unit of T4 DNA ligase (the concentrations were final concentrations). The mixture was kept cool at 16 °C for 15 hours to perform ligation of DNA.

By using the DNA after re-ligation, Escherichia coli K-12 strain (tryptophan-requiring; ATCC 23718) to obtain transformants [disappearance of trp-requirement]. By trpA, trpB genes on the plasmid, biosynthesis of tryptophan became possible and the transformants could be grown on a minimum medium (7 g of K_2HPO_4, 2 g of KH_2PO_4, 0.1 g of $MgSO_4.7H_2O$, 1 g of $(NH_2)_2SO_4$, 1 liter of deionized water)]. These transformants were cultivated in a liquid culture medium, and from the culture broth, a plasmid was separated and purified (this plasmid will be referred to as pBR322trp).

(D) Cloning of a fragment containing tryptophan synthase structural genes (trpA, trpB):-

The plasmid pBR322 trp obtained in section (C) was digested at 37 °C for 1 hour with restriction endonucleases SacII and SalI to obtain a fragment having a molecular size of about 3.5 kb and containing

trp A, trp B genes. Then, it was partially digested with restriction endonuclease HincII at 37 °C to obtain a minimum fragment having a molecular size of about 2.6 kb and containing trp A and trp B. This fragment Was mixed with a SalI linker, and ligated by using a T4 DNA ligase to give a fragment containing trp A and trp B and having a SalI site at both ends.

(2) Preparation of a DNA fragment containing a tryptophanase promoter and regulator gene tnaC located downstream of the promoter

(A) Preparation of chromosomal DNA of E. coli K-12 strain (ATCC27325)

100 ml of L-medium (10 g of Trypton, 5 g of yeast extraact, 1 g of glucose, 5 g of NaCl, 12 g of deionized water; pH 7.2) was put in a 500 ml Erlenmeyer flask, and sterilized at 120 °C for 15 minutes. E. coli K-12 strain (ATCC 27325) was inoculated in this medium. and cultivated at 37 °C for 15 hours. This culture broth (2 ml) was taken, and inoculated newly in 1000 ml of the above medium, and again cultivated at 37 °C for 4 hours.

After the cultivation, all the culture broth was centrifuged (8,000 rpm, 15 minutes, 4 °C) to harvest the cells. Then, the cells were suspended in 50 ml of 10 mM EDTA.2Na solution 50 mM Tris buffer (pH 8.0). Then, lysozyme was added to that its final concentration reached 2 mg/ml. After standing for 5 minutes, 6 ml of a 10 % solution of sodium dodecylsulfate was added, and the mixture was heated at 65 °C for 30 minutes and lyzed. To this lysis solution was added 15 ml of 5M NaCl solution, and the mixture was cooled at 0 °C for 1 hour. All the mixture was centrifuged (12,000 rpm, 60 minutes, 4 °C). The supernatant fraction was taken, and 6 times its weight of ethanol was added. The mixture was centrifuged (5,000 rpm, 10 minutes, 4 °C). The precipitate was dissolved in 10 mM Tris buffer (pH 7.5)-1 mM EDTA.2Na solution, and treated with phenol (protein-eliminating treatment) and with an RNA decomposition enzyme to finally give 1.5 mg of DNA.

(B) Preparation of a DNA fragment containing a tryptophanase promoter and a regulator gene tnaC located downstream of the promoter:-

The chromosomal DNA prepared in (A) above (25 micrograms) was digested at 30 °C for 1 hour with restriction endonucleases HindIII and BamHI (each 5 units). The digestion product solution was mixed with a digestion product solution obtained by digesting 1 microgram of plasmid pBR322 with 1 unit each of restriction endonucleases Hind III and BamHI at 39 °C for 1 hour. 50 mM Tris buffer (pH 7.6), 10 mM dithiothreitol, 1 mM ATP, 10 mM $MgCl_2$ and 1 unit of T4 DNA ligase (the concentration were final concentrations) were added to the mixture and reacted at 16 °C for 15 hours.

E. coli K-12 strain (tryptophanase-deficient mutant and tryptophan requiring mutant) was transformed in a customary manner [M. Mandel, A. Higa: J. Mol. Biol., 53, 159 (1970), and coated on a selective medium [7 g of K_2HPO_4, 2g of KH_2PO_2, 1 g of $(NH_4)_2SO_4$, 0.1 g of $MgSO_4.7H_2O$, 5 g of Casamino acid, 10 mg of indole, 2 g of adenine, 20 g of agar, 50 mg of succinic acid and 1 liter of deionized water]. The cells on the culture broth were inoculated on a culture medium obtained by adding ampicillin to the L-medium in a concentration of 50 micrograms/ml as a final concentration, and cultrivated at 37 °C for 7 hours. The cells were harvested by centrifugation (8.000 rpm, 10 minutes, 4 °C). The tryptophanase activity was examined using the harvested cells. To 1 ml of a reaction solution for measurement of the tryptophanase activity measurement (0.1M phosphate buffer pH 8.0; 0.10 mM tryptophan, 0.1 % Triton X-100) were added 50 mg of wet cells, and reacted at 37 °C for 30 minutes. The resulting indole was reacted with p-dimethylbenzaldehyde to give red-colored cells. The plasmid was extracted from these cells by using the alkali SDS method [T. Maniatis, E. F. Fritsch, J. Sambrook: "Molecular Cloning" (1982), see pages 90 to 91]. The plasmid was cleaved with restriction endonucleases BamHI and HindIII, and the molecular size was examined by using agarose gel. A DNA having a molecular size of about 3.2 kb was seen to be inserted into the HindIII and BamHI sites of the plasmid pBR322 (this plasmid will be referred to as pBR322tna).

The resulting pBR322tna was digested with restriction engonucleases AluI and RsaI at 37 °C for 1 hour to give a fragment having a molecular size of about 630 bp containing the tryptophanase promoter and the regulator gene tnaC. This fragment was mixed with a BamHI linker and ligated with T4DNA ligase to obtain fragment containing the tryptophanase promoter and the regulator gene tnaC and having a BamHI site at both ends.

(3) The DNA fragment obtained in section (1) above and the DNA fragment in section (2) above were inserted by a conventional method into the SalI site and BamHI site of plasmid pUC119 (a product of Takara Shuzo Co., Ltd.) Then, this plasmid was digested partially with restriction endonucleases EcoRI and HindIII to cut out a DNA fragment containing the tryptophanase promoter, the regenerator gene tnaC, and the tryptophan synthase structural gene (trp B, trp A). This DNA fragment was inserted in a customary manner into the EcoRI and HindIII sites of the plasmid pBR322. Furthermore, the mini-F fragment prepared in Example 1, (2) was inserted in a customary manner into the EcoRI site of this plasmid to prepare the desired plasmid.

(4) Transformation of E. coli K-12 strain with plasmid pMTY 20.

A tryptophanase-deficient strain was extracted from E. coli K-12 strain (ATCC 27325) by a conventional method ("Molecular Biology Experimental Manual" edited by Masaya Kawakami, page 20, 1984, published by Kodansha, Tokyo). This cell was used as a host and transformed with the plasmid DNA prepared in section (3) above. The transformants were cultivated in a customary manner in a liquid medium, and the plasmid was separated and purified. The resulting plasmid was named pMTY20.

The plasmid pMTY20 was digested with various restriction endonucleases, and the molecular sizes of the fragments were measured. The results are shown in Table 2.

Table 2

Restriction endonuclease	Number of recognition sites	Molcular weight (megadaltons; kb in the parentheses)
EcoRI	2	5.5(9.2), 4.4(7.4)
HindIII	3	5.6(9.3), 3.0(5.0) 1.3(2.3)
SalI	4	7.7(12.8), 1.4(2.4) 0.5(0.8), 0.3(0.6)
BamHI	5	4.1(6.8), 2.4(4.0) 1.6(2.7), 1.4(2.4) 0.4(0.7)

The restriction endonuclease map of the plasmid pMTY20 is shown in Fig. 2

Escherichia coli YK2017 transformed with the plasmid pMTY20 was deposited on November 17, 1989 at Fermentation Research Institue, Agency of Industrial Sciennce and Technology, Japan, 1-1-3, Higashi, Tsukuba-shi, Ibaraki-ken Japan under FERM P-11117 (transferred to international deposition FERM KBP-2804 on March 14, 1990 under the Butapest Treaty).

(5) Stability of the transformed Eschedrichia coli YK2017

100 ml of the above L-medium containing 2 g/liter of succinic acid in place of 1 g/liter of glucose as a carbon source was put in a 500 ml Erlenmeyer flask and sterilized at 120 °C for 15 minutes. The transformed cells Escherichia coli YK2017 obtained in section (4) above were inoculated in the L-medium, and cultivated under shaking at 37 °C for 24 hours. Then, 100 ml of a medium prepared in the same way as above was put in a 500 ml Erlenmeyer flask, and sterilized at 120 °C for 15 minutes. The cells were successively inoculated in the medium at a rate of 50 cells per ml, and cultivated at 37 °C for 24 hours under shaking.

Then, the cells were harvested by using centrifugation. The cells were washed, and then coated in a fixed amount on the L-medium to which ampicillin was added at a rate of 50 micrograms/ml and also on the L-medium to which no component was further added, and cultivated at 37 °C for 1 day. After the cultivation, the colonies which grew were counted.

It was found that the number of colonies which grew on the ampicillin-containing medium was the same as that which grew on the medium to which no other component was added. Furthermore, all colonies which grew on the L-medium grew on a minimum medium (containing 10 mg/liter of indole) containing no tryptophan. This led to the determination that the plasmid has high stability.

EXAMPLE 3

Preparation of plasmid pCAT-1 and the transformation of E. coli K-12 strain with the plasmid pCAT-1

(1) Preparation of plasmid pCAT-1

The about 630 bp DNA fragment having a BamHI site at both ends and containing the tryptophanase promoter and regulator gene tnaC located downstream of the promoter obtained in Example 2, (2) was inserted by a conventional method into the Bam HI site of plasmid pKK232-8 (purchased from Pharmacia Co., Ltd.) bearing a chloramphenicol acetyl transferase structural gene (about 0.66 kb) having no promoter of itself to prepare plasmid pKK232-8CAT. The mini-F DNA fragment (about 91 kg) obtained in Example 1, (2) was inserted into the Sma I site of the pKK 232-8CAT. This plasmid will be named as pCAT-1.

The plasmid pCAT-1 was cleaved with various restriction endonucleases. Table 4 shows the molecular sizes of the various cleaved fragments. The restriction cleavage map of the plasmid pCAT-1 is shown in Fig. 3.

Table 3

Restriction endonuclease	Number of recognition sites	Molcular weight (megadalton; kb in the parentheses)
EcoRI	3	6.6 (10.0), 3.1 (4.7), 0.07 (0.1)
Bam HI	3	7.67 (11.5), 1.7 (2.6), 0.4 (0.7)
Sal I	2	6.47 (9.8), 3.3 (5.5)

2) Transformation of E. coli K-12 strain with plasmid pCAT-1

Escherichia coli K-12 strain (ATCC 27325) was used as a host, and transformed in a customary manner by using plasmid pCAT-1 DNA prepared in Section (1) above. The resulting transformant was named Escherichia coli CAT-1.

(3) Stability of the transformant Escherichia coli CAT-1

100 ml of the above L-medium (containing 2 g/liter of succinic acid instead of 1 g/liter of glucose as a carbon source) was put in a 500 ml Erlenmeyer flask, and sterilized at 120 °C for 15 minutes. The transformants Escherichia coli CAT-1 obtained in section (2) were inoculated in the sterilized L-medium and then cultivated with shaking at 37 °C for 24 hours. Then, 100 ml of the same medium as prepared above was put in a 500 ml Erlenmeyer flask, and sterilized and the cultivation liquid was inoculated successively at a rate of 50 cells per ml, and cultivated with shaking at 37 °C for 24 hours.

The cells were harvested by centrifuging and washed. The cells were coated in a fixed amount on L-medium containing 50 micrograms/ml of ampicillin and in a plate medium prepared from L-medium without adding ampicillin, and cultivated at 37 °C for 1 day. The grown colonies were counted.

It was found that the number of colonies which grew was the same both for the ampicillin-containing medium and the medium not containing it; all colonies which can grow on an L-medium containing chloramphenicol at a rate of 50 micrograms/ml; namely, this plasmid had high stability.

EXAMPLE 4

production of tryptophanase by cultivating the transformant Escherichia coli YK3007

100 ml of the culture medium shown in Table 5 was put in a 500 ml Erlenmeyer flask and sterilized at 120 °C for 15 minutes. To the sterilized medium one milliliter of a 50 % (wt/vol) glucose solution (sterilized at 120 °C for 15 minutes) was aseptically added. Then, Escherichia coli YK3007 (FERM BP-2803), a tryptophananase-producing strain, was inoculated, and cultivated with shaking at 37 °C for 1 day. Twenty milliliters of the culture broth was inoculated in 1000 ml of a culture medium prepared so that the concentration of glucose reached the upper limit (wt/vol %) in each run shown in Table 5, and cultivated at 37 °C for 8 hours using an aeration stirred culture tank (6000 rpm, the amount of air passed 1 vvm, pH 7.2 adjusted with 28 % aqueous ammonia). The glucose was used as an 50 % (wt/vol) aqueous solution (sterilized at 120 °C for 15 minutes), and added aseptically continuously while periodically, the concentration of glucose in the culture liquid was measured so that the glucose concentration did not exceed the upper limit of the concentration in each run shown in Table 5 and did not fall below 0.01 % (wt/vol). The addition was stopped when the total amount of glucose added in each run reached 20 g.

Table 4

L-tryptophan	0.5 g
KH_2PO_4	1.6 g
K_2HPO_4	5.5 g
$(NH_4)_2SO_4$	3.0 g
$MgSO_4.7H_2O$	0.1 g
$FeSO_4.7H_2SO$	80 mg
Casamino acid (a product of Difco)	5.0 g
Distilled water	1000 ml
(adjusted to a pH of 7.2 with 28 % aqueous ammonia)	

After the end of cultivation, 20 ml fo the culture broth was centrifuged, and the activity of tryptophanase was measured by the method described above. The results are shown in Table 5. As a comparison, the cells cultivated by adding 20 g/liter of glucose at the start of the cultivation were used.

Table 5

Upper limit of the glucose concentration in the culture broth (wt/vol%)	Relative activity of tryptophanase (*)	Amount of the cells recovered (relative value *)
0.1	350	0.9
0.2	348	1.0
0.3	150	1.1
0.4	1.1	1.1
Comparison (glucose was added at the start of cultivation	1	1

(*) Relative value when the activity in the comparison (in which glucose was added in a concentration of 20 g/liter at the start of the cultivation) was taken as 1.

EXAMPLE 5

Production of tryptophan synthase by the cultivation of the transformant E. coli YK2017

One hundred milliliters of the culture medium shown in Table 4 in Example 4 was put in a 500 ml Erlenmeyer flask, and then sterilized at 120 °C for 15 minutes. One milliliter of a 50 % (wt/vol) glucose solution (steriliazed at 120 °C for 15 minutes) was aseptically added to the medium in the flask. Then, Escherichia coli YK2017 (FERM BP-2804), (tryptophan synthase-producing strain, was inoculated in the medium, and cultivated at 37 °C for 1 day. The culture broth (20 ml) was inoculated in 1000 ml of a culture medium prepared so that the concentration of glucose became the upper limit of the concentration (wt/vol %) in each run as shown in Table 6, and cultivated at 37 °C for 8 hours by using an aeration stirred cultivation tank (600 rpm, the amount of air passed 1 vvm, pH 7.2 adjusted with 28 % aqueous ammonia). The glucose was used as an 50 % (wt/vol %) aqueous solution (sterilized at 120 °C for 15 minutes), and was added aseptically continuously while periodically, the concentration of glucose in the culture broth was measured so that the glucose concentration did not exceed the upper limit of the concentration in each run shown in Table 6 and did not fall below 0.01 % (wt/vol). The addition was stopped when the total amount of glucose added in each run reached 20 g.

After the end of cultivation, 20 ml of the culture broth was centrifuged, and the activity of tryptophan-synthase was measured by the method described above. The results are shown in Table 6. As a comparison, the cells cultivated by adding 20 g/liter of glucose at the start of the cultivation were used.

Table 7

Upper limit of the glucose concentration in the culture broth (wt/vol)	Relative activity of tryptophan synthase (*)	Amount of the cells recovered (relative value *)
0.1	220	0.9
0.2	211	1.0
0.3	85	1.1
0.4	1.2	1.0
Comparison (glucose was added at the start of cultivation)	1.0	1.0

(*) Relative value when the activity in the comparison (in which glucose was added in a concentration of 20 g/liter at the start of the cultivation) was taken as 1.

EXAMPLE 6

Production of chloramphenicol acetyl transferase by the cultivation of transformant Escherichia coli CAT-1:-

One hundred milliliters of the culture medium shown in Table 4 in Example 5 was put in a 500 ml Erlenmeyer flask, and sterilized at 120 °C for 15 minutes. One milliliter of a 50 % (wt/vol) glucose solution (sterilized at 120 °C for 15 minutes) was aseptically added. Escherichia coli CAT-1 obtained in Example 3 was inoculated, and then cultivated at 37 °C for 1 day. Then, 20 ml of the culture broth was inoculatd in 100 ml of a culture medium prepared so that the concentration of glucose reached the upper limit in each run shown in Table 7, and cultivated by using an aeration stirred culture tank at 37 °C for 8 hours (600 rpm, the amount of air passed 1 vvm, pSH 7.2 adjusted by adding 28 % aqueous ammonia. The glucose was used as an 50 % (wt/vol %) aqueous solution (sterilized at 120 °C for 15 minutes), and was added aseptically continuously while periodically, the concentration of glucose in the culture broth was measured so that the glucose concentration did not exceed the upper limit of the concentration in each run shown in Table 6 and did not fall below 0.01 % (wt/vol). The addition was stopped when the total amount of glucose added in each run reached 20 g.

After the cultivation, 20 ml of the culture broth in each area was centrifuged, and the activity of chloramphenicol acetyl transferase was measured by the method described above. The results are shown in Table 7. As a comparison, the cells cultivated by adding 20 g/l of glucose at the start of cultivation were

used.

Table 8

Upper limit of the glucose concentration in the culture broth (wt/vol %)	Relative activity of chloramphenicol acetyl transferase (*)	Amount of the cells recovered (relative value *)
0.1	180	1.0
0.2	170	1.1
0.3	70	1.1
0.4	1.3	1.0
Comparison (glucose was added at the start of cultivation	1.0	1.0

(*) Relative value when the activity in the comparison (in which glucose was added in a concentration of 20 g/liter at the start of the cultivation) was taken as 1.

EXAMPLE 7

Production of tryptophan synthase by the cultivation of transformant E. coli YK2017

One hundred milliliter of the culture medium shown in Table 4 in Example r was put in a 500 ml Erlenmayer flask, and sterilized at 120 °C for 15 minutes, and 1 ml of a 50 % (wt/Vol) glucose solution (sterilized at 120 °C for 15 minutes) was aseptically added. Escherichia coli YK2017 (FERM BP-2804), a tryptophan synthase-producing strain, was inoculated in the resulting medium, and cultivated with shaking at 37 °C for 1 day. Then, 20 ml of the cultured broth was inoculated in 1000 ml of the same culture medium as prepared above, and cultivated at 37 °C for in an aeration stirried tank (600 rpm, the amount of air passed 1 vvm, pH 7.2 adjusted with 28 % aqueous ammonia). The glucose was used as a 50 % (wt/vol) aqueous solution (sterilized at 120 °C for 15 minutes). While periodically, the concentration of glucose in the culture broth was measured, glucose was continuously added aseptically so that its concentration did not exceed the upper limit in each run shown in Table 8. When the total amount of glucose in each test area reached 20 g, the addition was terminated.

The concentration of dissolved oxygen in the culture liquid was maintained in each of the values in each run shown in Table 8 by passing oxygen gas in place of air. In a system through which oxygen was not passed, the concentration of dissolved oxygen became 0 after about 4 hours.

After the cultivation, 20 ml of the cultivation liquid was centrifuged, and the activity of tryptophan synthase was measured by the method described above. As a comparison, the cells obtained by adding 20 g/liter of glucose at the start of the cultivation were used. The results are shown in Table 8.

Table 9

Upper limit of the glucose concentration (wt/vol, %)	Dissolved oxygen concentration of the culture broth (ppm)	Activity of tryptophan synthase (*2)	Amount of cells recvered (* relative value)
0.1	No control (*1)	220	1.0
	1	260	1.2
	8	265	1.3
0.2	No control	210	1.0
	1	250	1.2
	8	255	1.3
0.3	No control	85	1.1
	1	105	1.3
	8	110	1.4
0.4	No control	1.1	1.1
	1	1.3	1.3
	8	1.3	1.4
Comparison (*1)	No control	1	1.0
	1	1	1.2

*1: The cultivation was carried out by adding 20 g/liter of glucose at the start.

*2: relative values when the activity and the amount recovered of cells in the comparison were taken as 1.

*3: the concentration of dissolved oxygen was not controlled by passing through oxygen.

Claims

1. A process for cultivating a microorganism transformed with a recombinant plasmid at least containing (a) a DNA fragment containing a promoter and a regulator gene tnaC located downstream of the promoter in a tryptophanase operon (tna), and (b) a DNA fragment containing a desired structural gene which can express by the promoter, which comprises cultivating the transformed microorganism in a culture medium while adding glucose as a carbon source continuously or intermittently so that the concentration of glucose is maintained within the range of 0.01 to 0.3 %, and thereby allowing the desired structural gene to express in the microorganism.

2. The process of claim 1 in which the DNA fragment (a) is derived from a chromosomal DNA of Escherichia coli.

3. The process of claim 1 in which the DNA fragment (a) is a DNA fragment having a molecular size of about 630 bp prepared by digesting a chromosomal DNA of Escherichia coli with restriction endonucleases BamHI and HindIII to give a trptophan operon DNA fragment having a molecular size of about 3.2 kb, digisting said DNA fragment with AluI and RsaI.

4. The process of claim 1 in which the DNA fragment (b) is derived from Escherichia coli.

5. The process of claim 1 in which the DNA fragment (b) is a DNA fragment containing a tryptophanase structural gene (tna A), or a DNA fragment containing tryptophan synthase structural genes (trp A, trp B).

6. The process of claim 1 in a DNA fragment having both the DNA fragment (a) and the DNA fragment (b) is a DNA fragment obtained by digesting a chromosomaml DNA of Escherichia coli with restriction endonucleases BamHI and HindIII to give a tryptophanase operon DNA fragment, and deleting about 900 bp from the BamHI site of said DNA fragment.

7. The process of claim 1 in which the DNA fragment (b) is a DNA fragment having a molecular size of about 2.6 kb obtained by infecting a chromosomal DNA of Escherichia coli with phage ϕ (phi) 80 to induce a phage DNA taking up the tryptophan operon in the phage ϕ (phi) 80 DNA, digesting it with a restriction endonuclease BamHI to give a DNA fragment having a molecular size of about 20 kb, and further digesting it with restriction endonuclease HincII.

8. The process of claim 1 in which the recombinant plasmid is a plasmid holding

(a) a DNA fragment containing a promoter and a regulator gene (tnaC) located downstream of the promoter in a tryptophanase operon (tna),

(b) a DNA fragment containing the desired structural gene which can be expressed by the promoter,

(c) a DNA fragment containing a gene having the autonomic replicating ability of a Col EI-type plasmid, and

(d) a DNA fragment containing a gene encoding the plasmid partition system derived from an F plasmid.

9. The process of claim 8 in which the DNA fragment (c) is derived from plasmid pBR322.

10. The process of claim 8 in which the DNA fragment (d) is a mini-F fragment.

11. The process of claim 8 in which the recombinant plasmid is plasmid pMTP4, plasmid pMTP1, plasmid pMTP1R or plasmid pMTY20.

12. The process of claim 1 in which the microorganism is Escherichia coli.

13. The process of claim 1 in which the transformed microorganism is Escherichia coli YK3007 (FERM BP-2803), Escherichia coli K-12 YK3002 (FERM BP-1733), Escherichia coli K-12 YK3003 (FERM BP-1734) or Escherichia coli YK2017 (FERM BP-2804).

14. The process of claim 1 in which glucose is added to the culture medium continuously or intermittently so that its concentratioon is maintained at 0.002 to 0.25 % (wt/vol).

15. The process of claim 1 in which the concentration of L- or DL-tryptophan in the culture medium is 0.1 to 2 % (wt vol).

16. The process of claim 1 in which the cultivation is carried out under aerobic conditions.

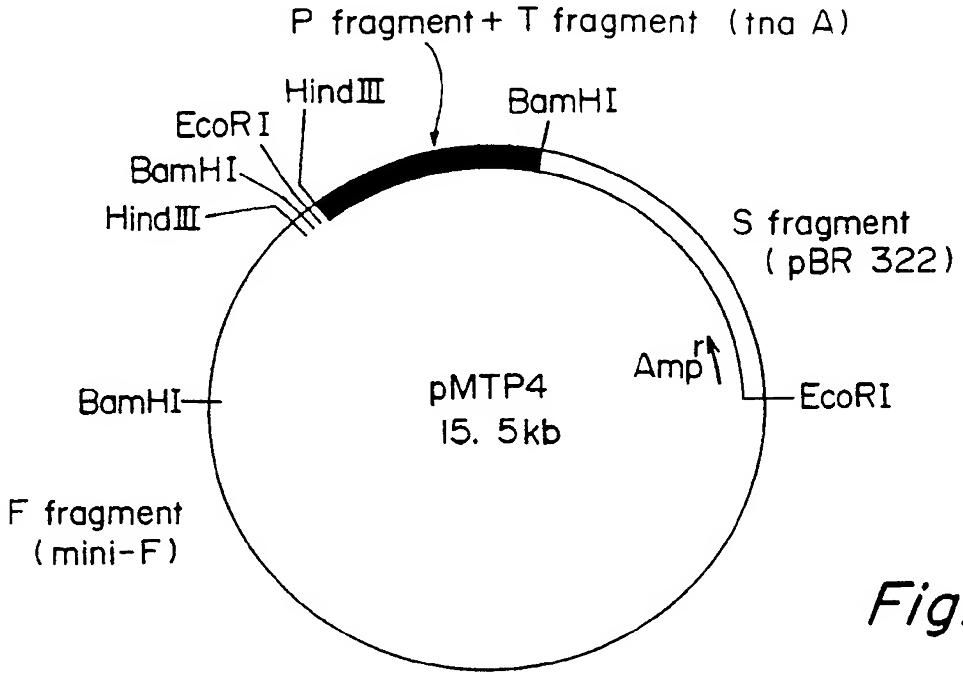
17. The process of claim 1 in which the cultivation is carried out while the concentration of dissolved oxygen in the culture medium is maintained at 1 to 8 pppm.

18. plasmid pMTP4.

19. Plasmid pMTY20.

20. Escherichia Coli YK3007 (FERM BP-2803).

21. Escherichia coli YK2017 (FERM BP-2804).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EcoRI
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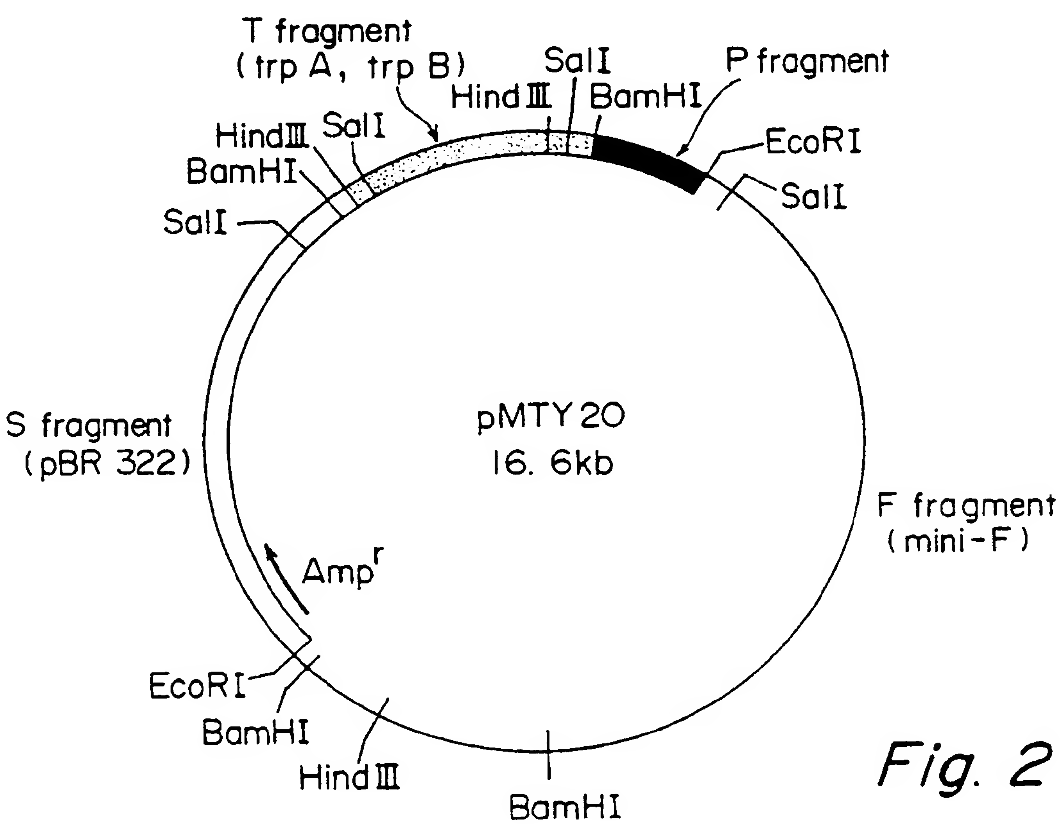
(mini-F)

Fig. 1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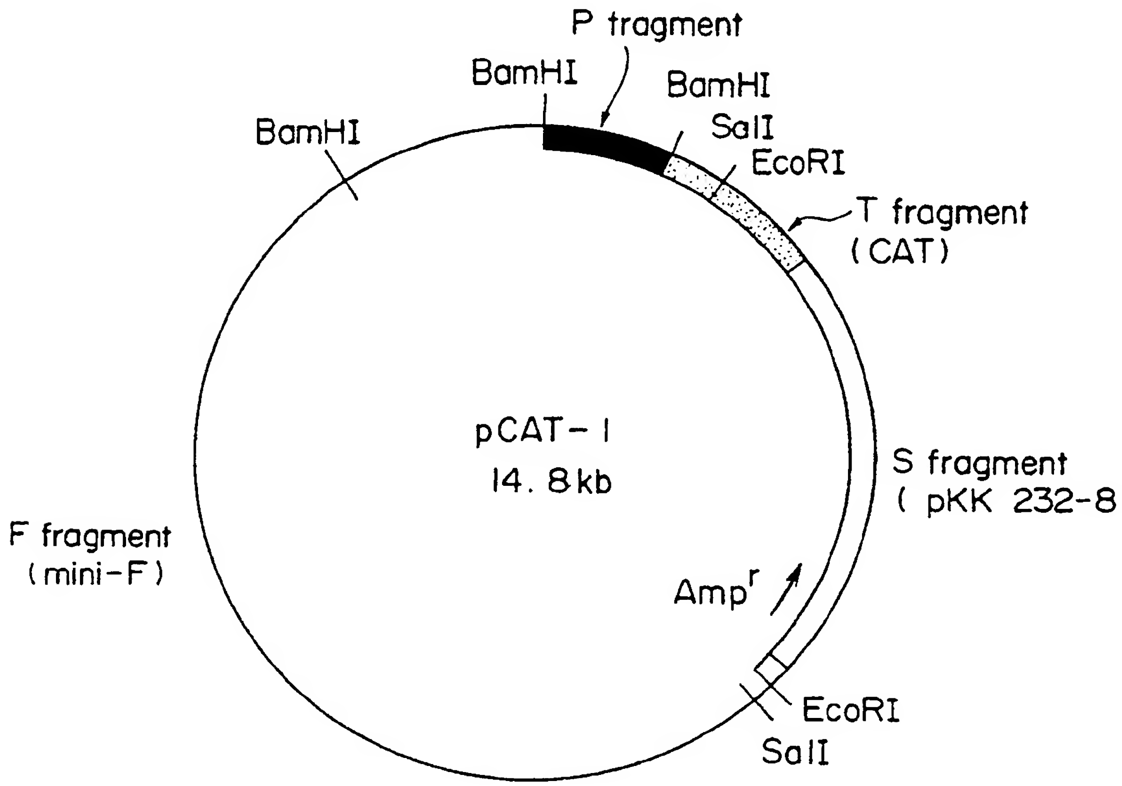
HindIII
BamHI

Fig. 2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F fragment
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Amp^r
EcoRI
SalI

Fig. 3

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 90 10 8609

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
X	PATENT ABSTRACTS OF JAPAN vol. 11, no. 85 (C-410)(2532) 14 March 1987, & JP-A-61 239896 (RES.ASSOC.UTIL.OF LIGHT OIL) 25 October 1986, * the whole document * ---	1, 5, 8, 10, 12	C12N15/60 C12P13/22 C12N9/88 C12N1/21 //(C12N1/21, C12R1:19)
Y	EP-A-281104 (RES.ASSOC.UTIL. OF LIGHT OIL) * page 5, lines 10 - 20 * * page 6, lines 39 - 50 * * page 9, lines 1 - 16; claims * ---	1-13, 16	
Y	EP-A-279664 (MITSUI TOATSU CHEMICALS, INC.) * abstract * * pages 3 - 5 * ---	1-13, 16	
A	EP-A-180192 (RES.ASSOC.UTIL. OF LIGHT OIL) * page 3, lines 5 - 29 * * page 4, line 34 - page 5, line 20 * * pages 25-26, example 6; page 28, example 9; page 31, example 13; claims* ---	1-10, 12, 14-16	TECHNICAL FIELDS SEARCHED (Int. Cl.5)
D,A	PATENT ABSTRACTS OF JAPAN vol. 12, no. 237 (C-509)(3084) 06 July 1988, & JP-A-63 28393 (RES.ASSOC.UTIL. OF LIGHT OIL) 06 February 1988, * the whole document * ---	1-2, 4, 8, 12	C12N C12P
A	CHEMICAL ABSTRACTS, vol. 102, no. 25, June 1985 Columbus, Ohio, USA MITSUBISHI PETROCHEMICAL Co.: "Plasmid pMIY-1" page 187; ref. no. 216308D * abstract * -----	1, 7	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	07 AUGUST 1990	ANDRES S.M.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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